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Synergistic and antagonistic effects of plant and dairy protein blends on the physicochemical stability of lycopene-loaded emulsions

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Kacie K.H.Y. Ho[ab], Karin Schroën[a], M. Fernanda San Martín-González[c], Claire C.Berton-Carabin[a]*

[a]Food Process Engineering Group, Department of Agrotechnology and Food Sciences, Wageningen University, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands

[b]Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Kannapolis, NC, 28081, USA

[c]Department of Food Science, College of Agriculture, Purdue University, 745 Agriculture Mall Drive, West Lafayette, IN, 47907, USA

*Corresponding Author

Highlights

- Whey-plant protein-based emulsions had high physicochemical stability.
- Whey and plant protein blend-based interfaces were viscoelastic while casein-based interfaces were relatively viscous.
- Whey-plant and plant-plant protein blends behaved synergistically leading to enhanced emulsion stability.
- Sodium caseinate-plant protein-based emulsions were physically unstable.

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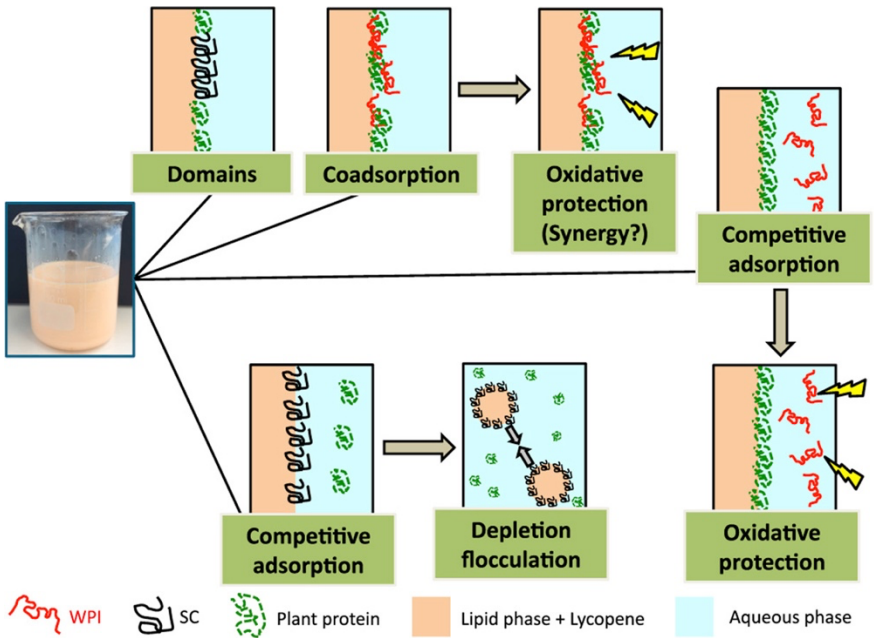
Graphical Abstract



1. Introduction

Oil-in-water (O/W) emulsions have been largely studied as delivery systems for lipophilic bioactive compounds, such as carotenoids (Cornacchia & Roos, 2011; Qian, Decker, Xiao, & McClements, 2013; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Lycopene, an unsaturated pigment from tomatoes, is the most hydrophobic and most potent singlet oxygen quencher of all carotenoids (Di Mascio, Kaiser, & Sies, 1989; Rao, Waseem, & Agarwal, 1998). However, lycopene is a hydrophobic compound that is highly susceptible to degradation, making it a challenging compound to incorporate into aqueous-based food systems.

Dairy proteins, such as sodium caseinate and β-lactoglobulin, are commonly used to stabilize O/W emulsions (Dalgleish, 1997; Dickinson, 1994, 2001; Hailing & Walstra, 1981; McClements, 2004). Plant proteins have not been studied as extensively, but are a sustainable alternative to animal-derived proteins. From a sustainability perspective, plant proteins (e.g., soy and pea proteins) require less resources for production than dairy proteins and are claimed to exhibit fair emulsion stabilization properties (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014; Liu & Tang, 2014). Soy proteins, which are primarily glycinin and conglycinin (Chronakis, 1996) and pea proteins, which are mainly legumin and vicillin (O'Kane, Vereijken, Happe, Gruppen, & J S Van Boekel, 2004), are globular proteins that have potential as food grade emulsifiers. We previously reported on the physicochemical stability of lycopene-loaded emulsions stabilized by dairy or plant protein (Ho, Schroën, San Martín-González, & Berton-Carabin, 2016). Overall, sodium caseinate (SC) and, to a lesser extent, pea protein isolate (PPI) could form physically and chemically stable emulsions. Conversely, using whey protein isolate (WPI) led to emulsions with poorly protected lycopene, and soy protein isolate (SPI)-based emulsions exhibited a significant increase in droplet size during storage.

The question we then posed was whether blending these proteins could lead to better physicochemical stability of lycopene-loaded emulsions, compared to the individual protein systems. Substantial work has already been done on the behavior of different dairy protein blends (e.g. WPI-SC) at the oil-water interface (Britten & Giroux, 1991; Dalgleish, Goff, & Luan, 2002; Seta, Baldino, Gabriele, Lupi, & de Cindio, 2012; Ye, 2008). Britten and Giroux (1991) observed preferential interfacial adsorption of SC from WPI-SC blends in O/W emulsions with 30% (w/w) oil phase. This preferential adsorption is due to the ability of SC to rapidly cover the interface (Seta, Baldino, Gabriele, Lupi, & Cindio, 2014). Dickinson (2011) has reviewed competitive adsorption in mixed biopolymer systems, which has been a topic of interest for many years for dairy protein blends and protein-polysaccharide blends. In mixed systems, SC is known to dominate the interface and dictate the interfacial behavior compared to less hydrophobic and non-flexible proteins, such as WPI.

Aoki, Shirase, Kato, and Watanabe (1984) found that SPI-SC blended emulsions were less stable compared to SPI-stabilized emulsions at ratios similar (1:1) and higher (4:1) than those used in this study. The authors hypothesized that this was likely due to rapid and preferential adsorption of SC to the interface. Other studies have indicated that blends containing SC and pea proteins can enhance emulsion physical stability compared to SPI or PPI alone due to a synergistic interaction between the flexible SC molecules and plant proteins (Ji et al., 2015) or their aggregates (Yerramilli, Longmore, & Ghosh, 2017). In particular, some proteins, e.g., β-

lactoglobulin, have relatively high packing densities and the ability to form strong protein-protein interactions (Dickinson, 2001), which could be beneficial. Considering this, we selected four proteins to blend: PPI, SPI, WPI and SC; the last one being an industrially proven standard. Our study aimed at assessing the effect of different interfacial dairy-plant protein blends on: 1) the physical stability (droplet size and surface charge) and 2) chemical stability (lycopene retention) of emulsions, and 3) the interfacial properties (adsorption kinetics and dilatational rheology). We ultimately attempted using these findings to propose hypotheses regarding protein blend behavior at the interface.

2. Materials and methods

2.1. Materials

All-*trans*-lycopene standard, all analytical grade solvents and reagents were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). Water was purified with an EMD Millipore Milli-Q water purification system and had a resistivity of 18.2 MΩ·cm. Proteins were generously donated as follows: WPI, 97.5% purity (BiPRO, Davisco, Switzerland), SC, 80% purity (Sodium caseinate S, DMV International, Amersfoort, the Netherlands), SPI, 90% purity (SUPRO EX 37, Solae Europe SA, Switzerland), and PPI, 80–90% purity (NUTRALYS F85, Roquette, France). More information on the solubility and properties in aqueous solution of the different proteins can be found in our previous work (Ho et al., 2016). Tomato paste, for lycopene extraction, and canola oil were purchased from local retailers. Canola oil was stripped of tocopherols and surface-active impurities via mixing with MP Alumina N-Super (MP Biomedicals, France) as described previously (Berton, Genot, & Ropers, 2011).

2.2. Methods

2.2.1. Lycopene extraction and oil phase preparation

Lycopene was extracted as previously described (Ho et al., 2016). Briefly, ~250 g of tomato paste, ~10 g of celite, ~10 g of sodium bicarbonate, and 500 mL of a 1:1 hexane (0.1% w/v butylated hydroxytoluene)-ethyl acetate mixture were stirred on ice under a stream of nitrogen, at 250 rpm with an overhead IKA mixer for 1.5 h. Following vacuum filtration, the liquid portion was washed with saturated sodium chloride solution before draining the bottom, aqueous layer from a separatory funnel. The organic portion of the liquid extract was collected, flushed with nitrogen and evaporated using a rotative agitator. Approximately 80 g of stripped canola oil were added to the dry lycopene extract, transferred to a borosilicate bottle, and held under a stream of nitrogen to ensure evaporation of residual solvent. This process was repeated several times and all resulting lycopene-oil batches were pooled prior to storage at −20 °C. Lycopene content was determined by measuring the absorbance of the extract at 471 nm using a UV-vis spectrophotometer [molar extinction coefficient of 1.85×10^5 $M^{-1}cm^{-1}$ (Britton, Liaaen-Jensen, & Pfander, 2004)]. Lycopene concentration in the stock oil was 0.24 mg/g oil. *Cis* and *trans* lycopene isomers were separated with high-performance liquid chromatography using a YMC Carotenoid S-3 C30 column (YMC America, Inc., Allentown, PA, USA) as previously described by Kean, Hamaker, and Ferruzzi (2008). Isomers were compared against an all-*trans*-lycopene standard (Ho, Ferruzzi, Liceaga, & San Martín-González, 2015) to determine the stock as ~90% all-*trans*-lycopene.

2.2.2. Aqueous phase preparation

Dairy proteins (WPI, SC) were solubilized in 0.01 M phosphate buffer, pH 7.0, overnight at room temperature using a magnetic stirrer (100 rpm). Unlike the dairy proteins, the commercial SPI and PPI proteins are only partially soluble (SPI~30%, PPI~25%) in buffer. Preliminary work with the commercial plant proteins indicated that emulsions stabilized with the whole protein fraction were polydispersed and had larger $d_{3,2}$ values (>1 μm). Since the interface is the site of oxidation (Berton-Carabin, Genot, Gaillard, Guibert, & Ropers, 2013) this study aimed at achieving similar droplet sizes for all emulsions to keep interfacial area comparable across all samples. SPI and PPI-based emulsions with comparable droplet sizes to WPI and SC-stabilized emulsions could only be achieved after removal of the insoluble protein fraction (Ho et al., 2016)

Prior to use, plant proteins were subjected to centrifugation (10,000×*g*, 10 min, 4 °C) after overnight stirring to remove the insoluble fraction. The soluble protein content in aqueous solutions was determined after centrifugation using the BCA protocol (Smith et al., 1985) at 562 nm using a DU 720 UV-vis spectrophotometer (Beckman Coulter, Woerden, Netherlands) prior to dilution with a 0.1 M phosphate buffer (pH = 7.0) to 5 g/L for SPI and 7 g/L for PPI. Protein concentrations were selected to allow for similar emulsion droplet sizes while limiting the amount of non-adsorbed protein in the aqueous phase as described previously (Ho et al., 2016), for which used an adapted protocol from Berton et al. (2011) was used. Briefly, emulsions were made using different protein solutions of different concentrations (1–10 g/L) using fabrication methods described in Section 2.2.3 and size analysis as outlined in section 2.2.4. Protein concentrations were selected for each individual protein so that non-adsorbed protein was not predominant, <30% in these samples (Berton et al., 2011), and droplet diameter was small and comparable for all samples (approximately 0.2 μm). This was done by measuring the droplet size ($d_{3,2}$) of emulsions with varying protein concentrations and comparing that with the percentage of non-adsorbed protein in the aqueous phase. The non-adsorbed protein was separated by centrifuging fresh emulsions at 1840×*g* for 1.5 h and then quantified using the BCA assay (Smith et al., 1985). Accordingly, the final concentrations of the protein solutions were 5 g/L for WPI, SC, and SPI, and 7 g/L for the PPI. When needed, protein solutions were combined and gently stirred (100 rpm) for 1 h prior to emulsification. This study focused on understanding the potential of plant proteins to replace or partially replace dairy proteins. Thus, protein blend were selected so that dairy protein (WPI or SC) was combined with plant protein (SPI or PPI). A 1:1 blend of SPI-PPI was also studied to observe if blending the two plant proteins could benefit emulsion stability. In addition to the 1:1 binary blends, two additional 3:1 blends were selected so that plant protein would mostly replace dairy protein in the system. The concentration and ratio combinations of the protein blend solutions used in this study are listed in Table 1. It should be noted that commercial plant proteins, SPI and PPI included, often exist in a highly aggregated state. Thus, although native SPI and PPI are primarily globular proteins, it is more appropriate to consider the plant protein solutions used in this study as solubilized SPI and PPI aggregates than distinct globular proteins. The physicochemical properties of the plant protein solutions were previously reported as Supplementary Information in our previous publication (Ho et al., 2016).

Table 1. List of protein combinations used in 1:1 and 3:1 aqueous blend solutions.

Ratio of Blend	Blend Name	Protein Solution 1	Protein Solution 2	Protein Concentration of Blend
1:1 Ratio (v/v)	SPI-WPI	SPI (5 g/L)	WPI (5 g/L)	5 g/L
	PPI-WPI	PPI (7 g/L)	WPI (5 g/L)	6 g/L
	SPI-SC	SPI (5 g/L)	SC (5 g/L)	5 g/L
	PPI-SC	PPI (7 g/L)	SC (5 g/L)	6 g/L
	SPI-PPI	SPI (5 g/L)	PPI (7 g/L)	6 g/L
3:1 Ratio (v/v)	SPI-WPI	SPI (5 g/L)	WPI (5 g/L)	5 g/L
	PPI-WPI	PPI (7 g/L)	WPI (5 g/L)	6.5 g/L

2.2.3. Emulsion preparation

Aliquots of frozen lycopene stock oil were thawed in an ultrasonic bath for 30 min, which allowed for lycopene crystal solubilisation in the oil. The aqueous phase and the oil phase (10% w/w) were combined and mixed with a rotor-stator homogenizer (UltraTurrax, IKA-Werke GmbH & Co., Staufen, Germany) at 11,000 rpm for 30 s to form a coarse emulsion, which was immediately passed through a high pressure M-110Y Microfluidizer (Microfluidics, Massachusetts, USA) at 800 bar, five times. The resulting fine emulsion was flushed with nitrogen and stored in borosilicate vials in the dark at 4 °C. Aliquots were taken from the emulsions at 0–14 days for physical characterization and lycopene stability. Those used for lycopene quantification were stored in the dark in glass vials, flushed with nitrogen, and held at −20 °C until the samples were extracted and analyzed.

2.2.4. Physical characterization of emulsions

2.2.4.1. Droplet size measurement

Emulsion droplet size was determined using a static light scattering instrument (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Droplet size was reported as the mean ($d_{3,2}$) and was measured within an obscuration range of 12–16%. The refractive indices applied for the continuous and dispersed phases were 1.330 and 1.475, respectively. For emulsions that were suspect of flocculation, aliquots were diluted (1:4 w/w) in a 10% sodium dodecylsulfate (SDS) solution prior to measurement to determine the size of the individual droplets.

2.2.4.2. Zeta potential measurement

Emulsions were diluted with ultrapure water to a dispersed phase fraction of 0.122% (v/v) and analyzed at 25 °C following a 2 min equilibration period. The electrophoretic mobility of droplets was measured via laser Doppler velocimetry using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) with a backscatter angle of 173°. Zeta potential was calculated by the Malvern software using the Smoluchowski model (refractive indices of the

continuous and dispersed phases 1.330 and 1.475, respectively). Zeta potential values were expressed as the mean of three independent samples, of which each was measured in triplicate.

2.2.5. Determination of lycopene retention in emulsions

Lycopene was extracted from emulsions using a method developed by Ax, Mayer-Miebach, Link, Schuchmann, and Schubert (2003) with modifications (Ho et al., 2016). Emulsion aliquots (1 mL) were combined with 4 mL of hexane containing 0.1% BHT (w/v), 3 mL of ethanol, and 1 mL of saturated sodium chloride in water. The mixture was vortexed, held under a stream of nitrogen, and then placed into an ultrasonic bath for 5 min. The organic solvent layer was carefully removed with a Pasteur pipette while the remaining liquid was re-extracted with fresh solvent 3 additional times. The extracts from each sample were pooled and then diluted appropriately to achieve an absorbance value between 0.1 and 0.8. Lycopene was quantified as described in section 2.2.1 and was expressed as a relative retention value (%), which is defined as follows (Eq. (1)):

$$(1)\text{Lycopene retention (\%)} = (C_t/C_0) \times 100$$

Where C_0 is the initial lycopene content (mg/100 g of emulsion) and C_t is the lycopene content at time t. Encapsulation efficiency was defined as C_0, compared against C_i, the amount of lycopene added (Eq. (2)):

$$(2)\text{Encapsulation efficiency (\%)} = (C_0/C_i) \times 100$$

To prevent carotenoid degradation during analysis, samples were held on ice, extracted in the dark, and flushed with nitrogen to limit exposure to heat, light, and oxygen.

2.2.6. Protein film behavior at the oil-water interface

2.2.6.1. Adsorption kinetics

An automated drop tensiometer (Teclis, Longessaigne, France) was used to measure the interfacial tension at the oil-water interface. The experimental set-up consisted of a model oil drop (stripped oil) in a glass cuvette (Hellma Analytics, Jena, Germany) containing an aqueous protein blend (0.1 g/L) in phosphate buffer (10 mM, pH 7.0). Lycopene was not included in the oil drop because preliminary experiments indicated that added lycopene did not have any effect on adsorption kinetics compared to lycopene-free stripped oil, which is explained by its high lipophilicity that keeps the component away from the interface (data not shown). Each model oil drop (60 mm^2 surface area) was formed with a 0.5 mL glass syringe, which was connected to a 16-gauge stainless steel needle. Interfacial tension, calculated via the Young-Laplace equation, was recorded for 2 h. Between sample runs, cuvettes, needles, and syringes were profusely cleaned with ethanol, 1% detergent solution (Hellmanex, Hellma Analytics, Jena, Germany), and ultrapure water.

2.2.6.2. Interfacial rheology

For each individual protein and protein-mixture, dilatational deformation sweeps were performed with the drop tensiometer following the 2 h equilibration period described in the previous section. The model drop was subjected to amplitude sweeps in which the drop was compressed and expanded to 2–35% of its original area (60 mm^2) with a constant frequency (0.01 Hz). The dilatational elastic (E'_d) and loss (E''_d) moduli were calculated as follows (Eqs. (3), (4)):

$$(3) E'_d = \Delta\gamma \, (A_0/\Delta A) \cos\delta$$

$$(4) E''_d = \Delta\gamma \, (A_0/\Delta A) \sin\delta$$

where $\Delta\gamma$ is the change in interfacial tension, A_0 is the initial drop surface area, ΔA is the change in drop surface area during the oscillations, and δ is the phase shift.

The loss tangent ($\tan\theta$) was calculated and expressed as a function of the applied deformation ($(A-A_0)/A_0$) (Eq. (5)):

$$(5) \tan\theta = E''_d / E'_d$$

A limitation of using the first harmonic Fourier transform, which was used to determine the elastic and loss moduli, is that any nonlinearities present in the raw signal are disregarded (Ewoldt, Hosoi, & McKinley, 2007; Van Kempen, Schols, Van Der Linden, & Sagis, 2013). Lissajous-Bowditch plots can thus be used to analyze complex interfaces that experience non-linear interfacial tension response upon compression and expansion. For this, the surface pressure is plotted as a function of deformation. The resulting plot shape can be related to film rheology (Fig. 1), but more importantly, asymmetries in shape can be indicative of a complex interface (Sagis & Scholten, 2014) with e.g., distinct protein domains.

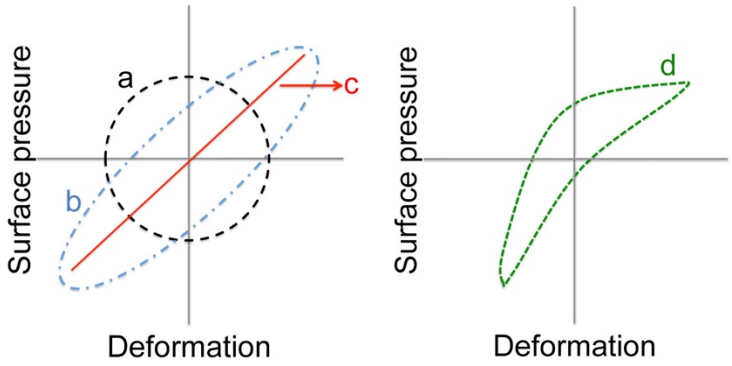


Fig. 1. Examples of Lissajous-Bowditch curves depicting viscous (a), viscoelastic (b), elastic (c), and non-linear viscoelastic (d) interfaces. Figure adapted from Deshpande (2010) and Sagis and Scholten (2014).

2.2.7. Statistical analysis

Values obtained for physical and chemical stability represent the mean and standard deviation from three independently prepared emulsions. JMP version 11 (SAS Institute Inc., Cary NC, USA) was used to statistically analyze data with one-way ANOVA and to compare means post-hoc with the Tukey-Kramer method. In all statistical analyses, significance was established with $\alpha = 0.05$.

3. Results and discussion

3.1. Physical stability of lycopene-loaded emulsions

All fresh emulsions showed fairly similar droplet diameters ($d_{3,2}$), between 0.18 and 0.21 μm. During the 14-day storage period, most emulsions were macroscopically stable and did not show creaming or phase separation (Fig. 2), which was confirmed by static light scattering measurements (Fig. 3A, B, C, D, G). The emulsions stabilized with PPI-WPI blends exhibited very good physical stability, as the droplet size distribution remained unchanged over time and regardless of dilution with SDS (Fig. 3B, D), which indicates that the samples were not susceptible to coalescence or flocculation. The emulsion stabilized with the SPI-PPI blend (Fig. 3G) also showed good physical stability, with the exception of a slight shift towards a right skewed distribution at day 14 without SDS dilution, indicating minor and reversible flocculation. Emulsions stabilized with the 1:1 and 3:1 SPI-WPI blends (Fig. 3A, C) were probably slightly flocculated, as indicated by a minor droplet population visible around 1–10 μm, that disappeared when the samples were diluted in SDS solution.

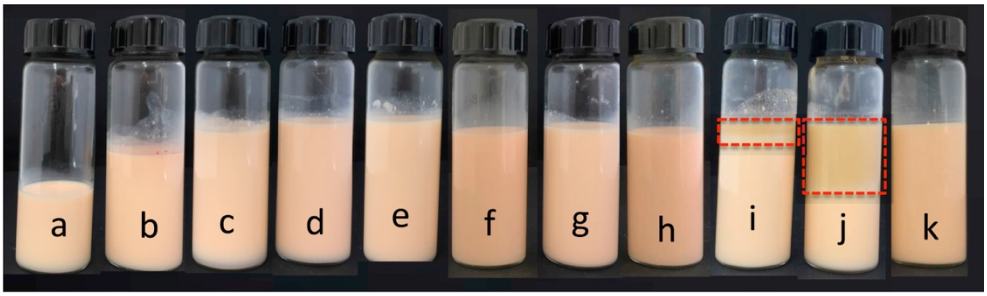


Fig. 2. Visual appearance of lycopene-loaded emulsions stabilized by WPI (a), SC (b), SPI (c), PPI (d) or protein blends, 1:1 SPI-WPI (e), 3:1 SPI-WPI (f), 1:1 PPI-WPI (g), 3:1 PPI-WPI (h), 1:1 SPI-SC (i), 1:1 PPI-SC (j), 1:1 SPI-PPI (k) on day 14 of storage. SC-blend samples (i, j) exhibit a lighter color compared to the other emulsions and an orange creamed layer (highlighted in the dashed line box).

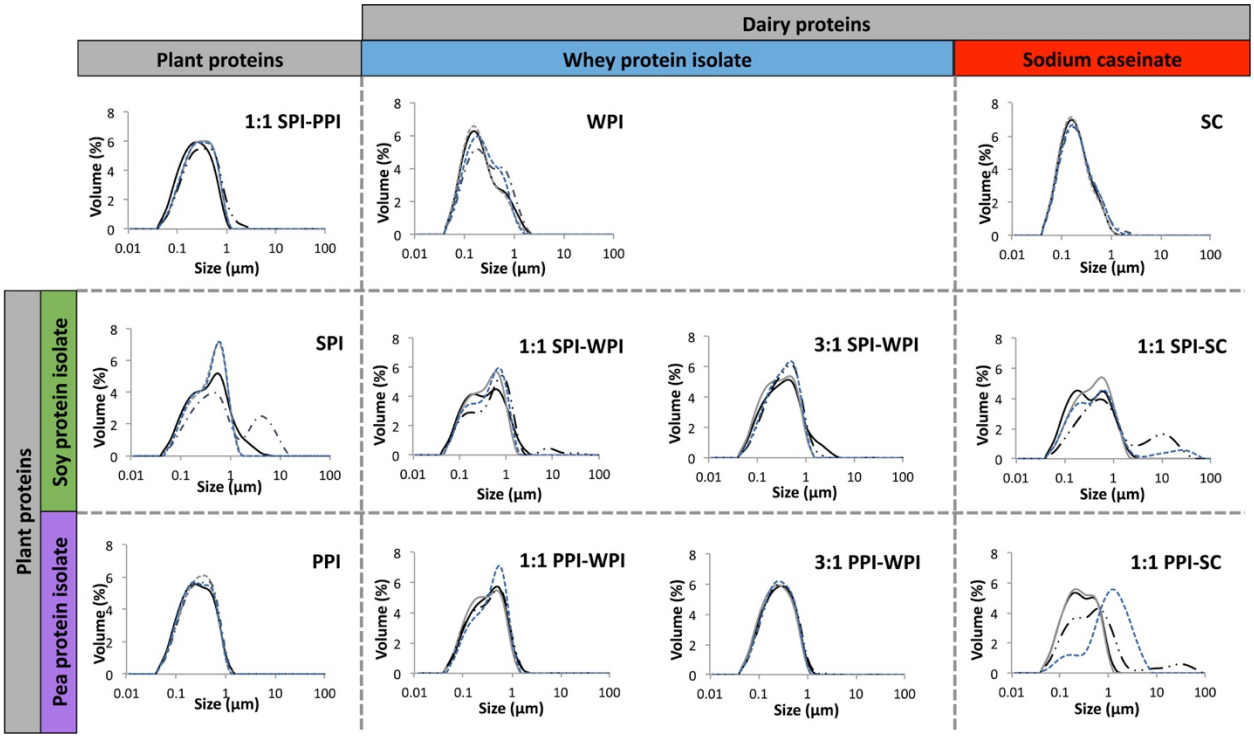


Fig. 3. Comparison of droplet size distributions of lycopene-loaded emulsions stabilized with individual proteins and protein blends at day 0 (thick black line), day 0 with 1% SDS (thin black line), day 14 (dashed black line), and day 14 with 1% SDS (dashed blue line). Identical distributions with and without SDS dilution suggest that flocculation did not occur. When Day 0 and Day 14 distributions are identical the emulsions are physically stable.

Contrary to the stable WPI-blends, SC-blends seemed to induce antagonistic effects on emulsion stability. These samples showed macroscopic phase separation and exhibited a visible creamed layer after 14 days of incubation (Fig. 2). The emulsion stabilized with the 1:1 PPI-SC blend was the least stable and exhibited a dramatic increase in droplet diameter from ~0.18 to 1.18 μm by day 14 (Fig. 4). Although the emulsion droplets stabilized with the SPI-SC blend did not appear to grow as dramatically, the span significantly increased from ~3.4 on day 7–~11.6 by day 14 (Table A.1, Supplementary Information). These effects were to some extent caused by flocculation, as can be deduced from the distributions obtained after dilution with SDS that have lower average droplet size, but coalescence did take place since the droplet size distribution no longer coincided with the one measured initially (Fig. 3E and F). The dramatic changes in droplet sizes for SC-blends imply that there are antagonistic effects on physical stability when these plant proteins are blended with SC.

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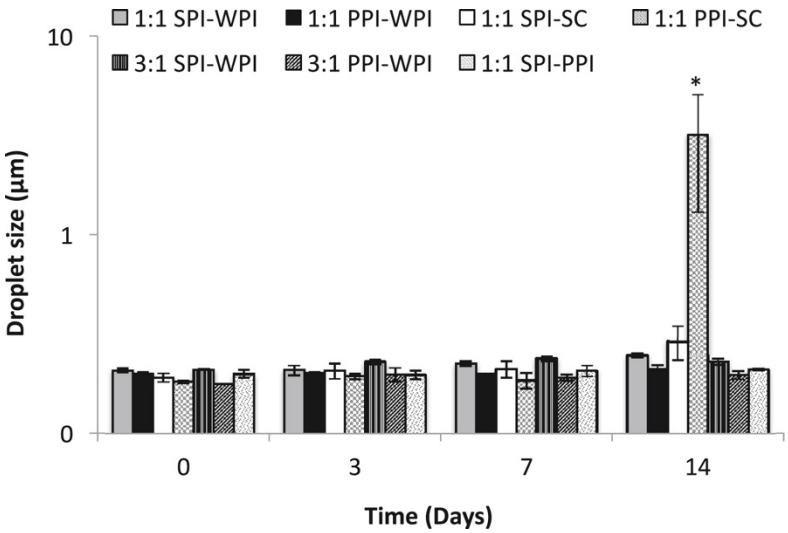

Fig. 4. Droplet size, $d_{3,2}$ of lycopene-loaded emulsions over time. Response values shown represent the mean ± standard deviation (n = 3). At day 14, an asterisk (*) denotes a value that is significantly ($p > 0.05$) different. Statistical differences for values at days all other time points (days 0, 3, and 7) are listed in Table A.1.

Although our study suggests antagonistic effects, other research groups have reported improved emulsion stability when SC was blended with either SPI or PPI. Yerramilli et al. (2017) observed an improvement in emulsion stability when using blends of PPI and SC and hypothesized that the SC molecules were able to surround excess PPI to limit depletion flocculation and to better solubilize PPI in the aqueous phase (Yerramilli et al., 2017). Similarly, Ji et al. (2015) observed a synergistic effect for SC and SPI which resulted in stable emulsions, while the individual proteins did not. The differences between these previously reported findings and the results of this study could be due to the selected protein concentrations: <1% (w/w) to limit non-adsorbed protein in the current work, while both Ji et al. (2015) and Yerramilli et al. (2017) worked with higher protein concentrations (2% and 5–10%, respectively) and likely had larger proportions of non-adsorbed protein in the aqueous phase. In our study, only the soluble protein fraction was used because this led to more stable emulsions than using the whole protein isolate suspension (including the insoluble fraction) (Ho et al., 2016). In contrast, Yerramilli et al. (2017) used a pre-homogenization step to break aggregates and improve dispersibility. Both protein concentration and solubility/presence of insoluble fraction may thus have influenced emulsion stability. When considering the differences in results amongst research groups, it is thus critical to consider the chosen proteins, concentrations, and pretreatments as these greatly impact the nature and functionality of proteins in the emulsion system.

The zeta potential of different emulsions stabilized by protein blends was and remained largely negative over 14 days for all samples (Fig. 5; pH 7 > IEP proteins), which is in line with our previous results for the individual WPI, SC, SPI, and PPI (Ho et al., 2016). It is important to note that the electrostatic charge of emulsion droplets is not necessarily indicative of the emulsions' physical stability; for protein-stabilized emulsions, the formation of thick, viscoelastic interfacial films is expected to be a major factor in that respect, which is further investigated in section 3.3.

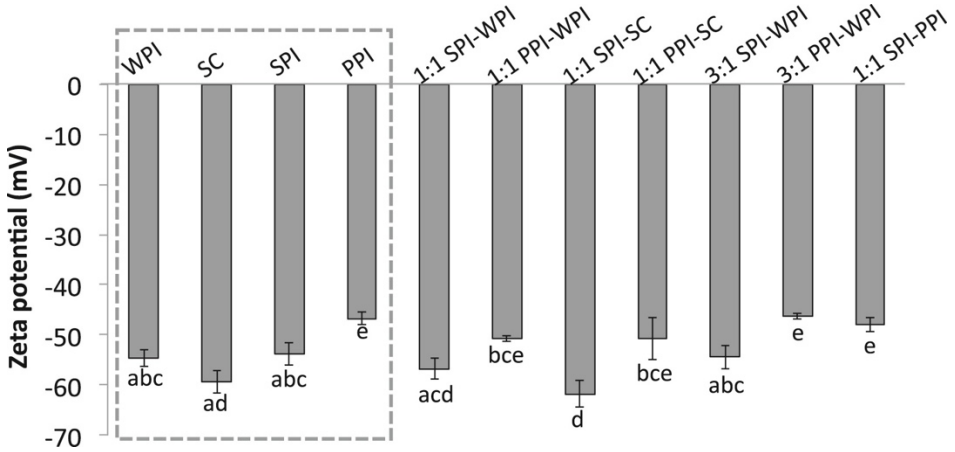

Fig. 5. Initial zeta potential of lycopene-loaded emulsions fabricated with proteins and protein blends. The results previously obtained for emulsions stabilized with the individual proteins are displayed as reference (within the gray dashed-line box), while the results obtained for emulsions stabilized with protein blends are shown to the right. Data shown represent the mean ± standard deviation (n = 3), with same superscript letters denoting values that are not significantly different ($p > 0.05$).

3.2. Chemical stability of lycopene in emulsions

Lycopene retention was expressed as a percentage since the initial encapsulation efficiencies (Table A.2) varied slightly amongst samples. Encapsulation efficiencies for all emulsions were between ~47 and 63%, with the efficiencies of the emulsions stabilized with the blends being statistically similar to that of at least one of the emulsions stabilized with the individual protein components. This suggests that blending plant and dairy protein does not significantly change the initial encapsulation efficiency compared to using one protein alone.

Due to the physical destabilization of the emulsions stabilized with SC-based blends, these samples were not measured although their lycopene retention at day 3 was lower than that of SC and all other blends at day 14 as reported in the supplementary information (Table A.3). All other emulsions exhibited at least 60% retention of lycopene after 14 days of storage, with protein blend-stabilized emulsions retaining >80% (Fig. 6). Overall, emulsions stabilized by WPI in combination with a plant protein (in particular, the 1:1 blends SPI-WPI and PPI-WPI), showed a higher lycopene retention over time compared to emulsions stabilized with the individual protein counterparts. When both plant proteins were used as a blend (1:1 SPI-PPI), the lycopene retention was one of the highest observed. These results suggest that blending plant protein with either WPI or another plant protein can improve the chemical stability of lycopene-loaded emulsions compared to using WPI alone. Additionally, compared to SC, the best performing individual protein, blends containing plant proteins performed statistically similarly or better with regards to chemical stability.

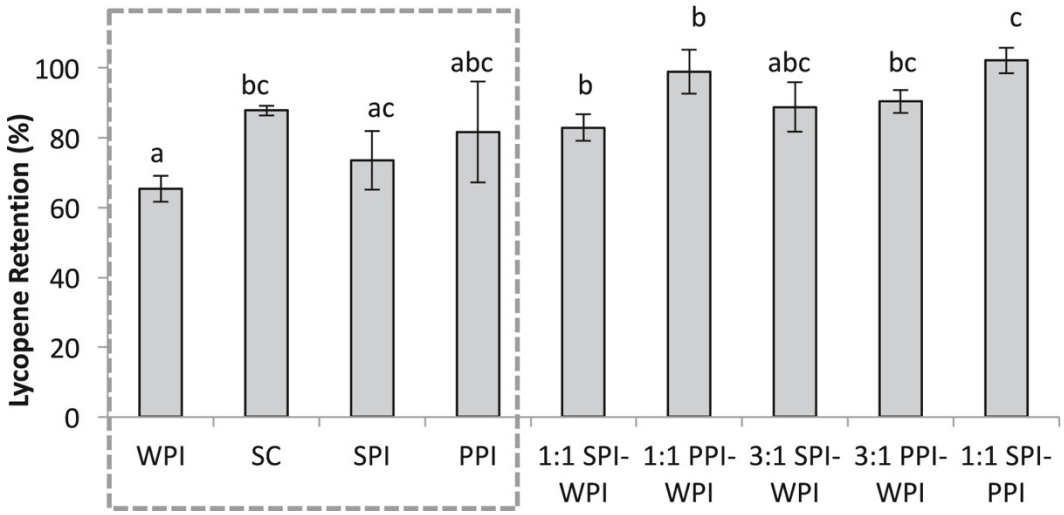

Fig. 6. Percent lycopene retention after 14 days of storage. The results previously obtained for emulsions stabilized with the individual proteins are displayed as reference (within the gray dashed-line box), while the results obtained for emulsions stabilized with protein blends are shown to the right. Values shown represent the mean ± standard deviation (n = 3), with same superscript letters denoting values that are not significantly different ($p > 0.05$).

Although SC has been shown to outperform WPI and plant proteins with regards to the chemical stabilization of lipophilic ingredients in emulsions (Ho et al., 2016; Hu, McClements, & Decker, 2003), some of the protein blend-based emulsions presented here had lycopene retentions that were similar to SC, which suggests that plant protein blends could be used as an alternative. This can be related to many proteins, including WPI, SC, SPI, and PPI, having antioxidant properties (Han & Baik, 2008; Mcgookin & Augustin, 1991; Peng, Xiong, & Kong, 2009; Peña-Ramos & Xiong, 2003), e.g. exposure of free-radical scavenging residues were reported for β-lactoglobulin (Elias, Kellerby, & Decker, 2008). As mentioned in Section 2.2.2, protein concentrations in this study were selected to limit the non-adsorbed protein (<30% excess protein). However, it is also important to note that some amount of protein still existed in the aqueous phase and may have influenced the lycopene stability through their antioxidant action (Berton-Carabin, Ropers, & Genot, 2014; Elias, McClements, & Decker, 2005; Faraji, Mcclements, & Decker, 2004; Min Hu, Julian McClements, & Decker, 2003).

3.3. Properties of protein layers at the oil-water interface

3.3.1. Adsorption kinetics

Fig. 7A distinguishes the commonly described steps for protein adsorption: (i) an initial induction period, corresponding to diffusion of proteins to the interface; (ii) a steep decline in the interfacial tension, corresponding to the interface becoming filled; and (iii) a slow decline in interfacial tension, corresponding to conformational changes of the adsorbed layer (Beverung, Radke, & Blanch, 1999). For all tested individual proteins and protein blends, equilibrium interfacial tensions at the stripped oil-water interface were ∼15–16 mN/m and were not largely

different except for WPI, which was slightly less surface-active with an equilibrium value of ~18 mN/m.

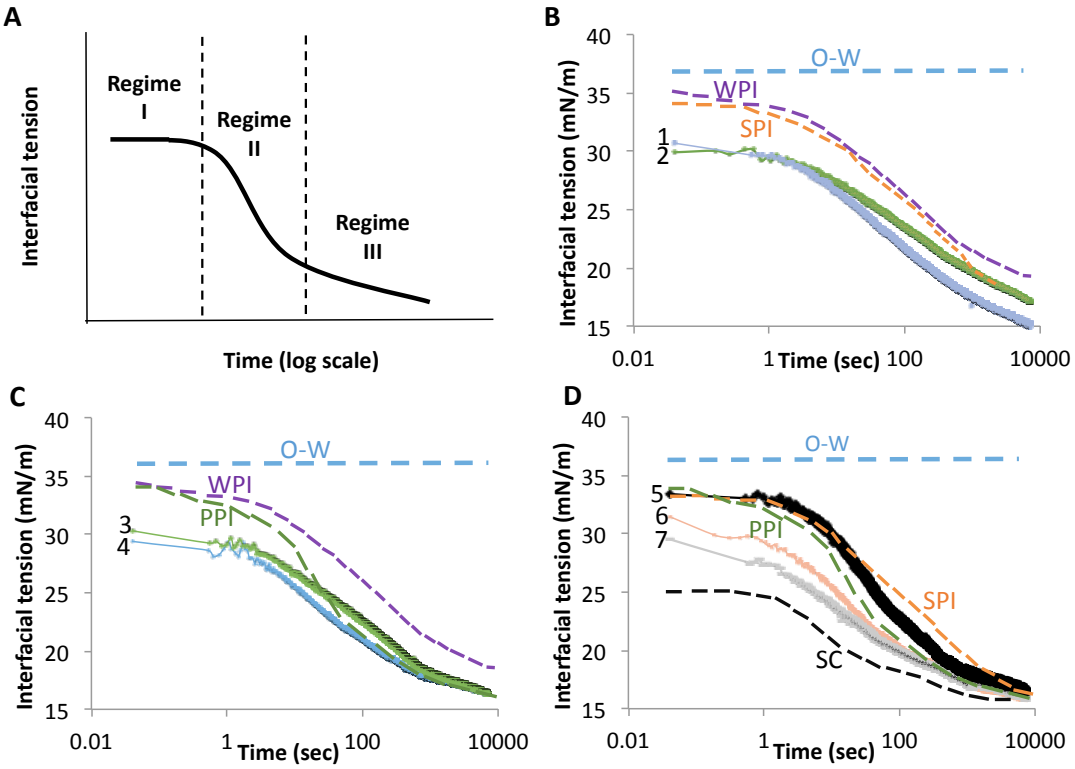

Fig. 7. Adsorption kinetics of the three stages of adsorption, typically expected for the dynamic interfacial tension response of proteins (A) is shown as an example adapted from Beverung et al. (1999) and the observed adsorption kinetics for SPI and WPI (B), PPI and WPI (C), and SC and plant protein blends (D) at the O-W interface. The interfacial tension as function of time for 3:1 SPI-WPI (1), 1:1 SPI-WPI (2), 3:1 PPI-WPI (3), 1:1 PPI-WPI (4), 1:1 SPI-PPI (5), 1:1 SPI-SC (6), and 1:1 PPI-SC (7) are plotted as data points connected by solid lines. Dashed lines correspond to individual proteins (WPI, SPI, PPI, and SC) and the interfacial tension of oil-water interface is represented by a blue dashed horizontal line for reference.

WPI-based blends appeared to reach the equilibrium interfacial tension faster than WPI alone, and also systematically showed a lower first measurement point, indicative of fast (sub-second) adsorption of material (Fig. 7B and C). Although plant proteins, in particular PPI, appeared to have a faster rate of adsorption than that of WPI, the WPI-based blends exhibited an even more immediate drop in interfacial tension. β-lactoglobulin has the potential to bind hydrophobic compounds (Kontopidis, Holt, & Sawyer, 2004) and potentially interact with other macromolecules (Flower, 1996). Previously, Mackie, Husband, Holt, and Wilde (1999) found that adsorption of genetic β-lactoglobulin variants, differing by only two amino acids, had substantially different adsorption behaviour at the air-water interface. Thus, even slight differences in structure could have a major effect on adsorption kinetics. However, in the present study, it is not clear why certain blends exhibited faster adsorption compared to individual proteins. Further work would be needed to assess if there are interactions or aggregation between WPI and the soluble SPI or PPI fractions that could favor early adsorption.

In the early stages of adsorption (t < 1 s) the interfacial tension of SC-based blends is substantially lower than that of SPI or PPI (Fig. 7D). This could be indicative of SC preferentially adsorbing and dominating the interface. Britten and Giroux (1991) observed that interfacial tension of casein-whey composite blends was dominated by casein due to its preferential adsorption at the interface. Seta et al. (2014) studied β-lactoglobulin and β-casein mixed layer films at the oil-water interface and observed that adsorption kinetics of mixtures fell between those of the individual proteins. In both studies, it was noted that there appeared to be a greater influence of casein, and this could be due to the flexible nature of the random coil protein that allows SC to rapidly unfold and adsorb at the interface (Alan R. Mackie, Gunning, Wilde, & Morris, 2000; Mitchell, 1986). For the 1:1 SPI-PPI blend, the adsorption behavior was close to both SPI and PPI (Fig. 7D), so it is difficult to conclude how the blend behavior is related to its individual counterparts.

Interestingly, plant protein blends, which exhibited a faster drop in interfacial tension compared to individual WPI and plant proteins (Fig. 7) also led to better emulsion stability against flocculation (Fig. 3) compared to the individual proteins (Ho et al., 2016). However, clear links between the adsorption kinetics and emulsion stability cannot be concluded as the $d_{3,2}$ values of the WPI- and plant protein blend-based emulsions (Fig. 4) did not seem substantially different from the emulsions made with individual proteins, which were reported previously (Ho et al., 2016).

3.3.2. Interfacial rheology

The rheological properties of the interfacial protein-based films can provide insight into emulsion stability to coalescence, as the film rupture between two emulsion droplets can be seen as dilatational deformation (Bos & van Vliet, 2001; Murray, 2011). Although rheological properties of the interface do not necessarily prevent flocculation, those properties can affect whether droplets reversibly flocculate or irreversibly coalesce, as coalescence can be seen as local dilatational deformation of the interface. With the exception of WPI, the elastic and loss moduli of all samples did not appear to have a major dependence on the applied deformation (Fig. 8A and B), which suggests that the measurements were mostly conducted in the linear viscoelastic regime, as was the case for the individual proteins (WPI, SC, SPI, PPI; Ho et al., 2016). Of all the proteins measured, SC clearly showed the lowest E_d' and highest loss tangent. The random coil structure and flexible behavior of SC contributes to its ability to sterically stabilize droplets (Nylander, 1998) but limit its ability to pack tightly at the interface (Dickinson, 2001) with adjacent SC proteins being unable to form strong intermolecular protein interactions and interfacial network. Except for the 1:1 PPI-SC mixture, all blends showed tangents in between those of the individual constituent proteins and relatively elastic behavior compared to SC (Fig. 8C), which suggests that the interface elasticity is determined by contributions from both proteins, and is most likely due to the ability of the proteins to form viscoelastic films via intermolecular interactions (Chang, Tu, Ghosh, & Nickerson, 2015; Dickinson, 2001).

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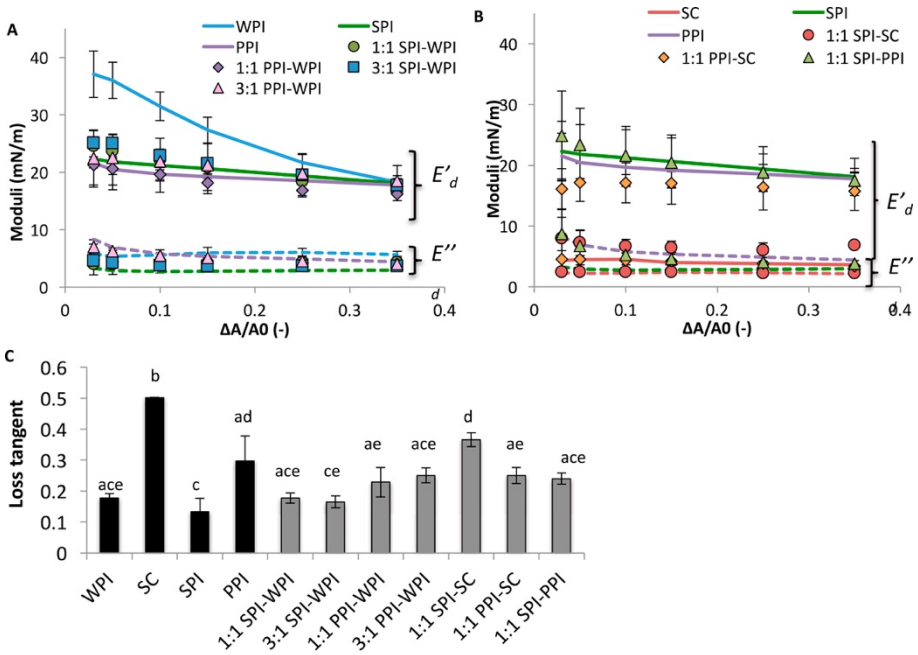

Fig. 8. Comparison of (A) WPI blends and (B) SC blends + SPI-PPI blend elastic moduli, E'_d (a) and loss moduli, E''_d (b) for protein films at the oil–water interface; and (C) loss tangent (E''_d/E'_d) at 0.15 deformation. Results for individual proteins are shown as: (A, B) solid lines (E'_d), dashed lines (E''_d) and (C) black bars for reference. Data shown represent mean ± standard deviation (n = 3). Statistical differences amongst protein films are shown (C) with same letters denoting values that are not significantly different (α = 0.05).

When plotting the data as Lissajous-Bowditch curves, more information regarding the interfacial behavior can be obtained (Sagis & Fischer, 2014). At low deformation of 0.1 the plots appear symmetric in shape (Fig. 9), meaning that linear interfacial behavior was observed. Although not observed at low deformations, an asymmetric plot indicates nonlinear interfacial behaviour, such as hardening in compression or softening in expansion (Sagis & Fischer, 2014). The film made of the 1:1 SPI-SC blend exhibited a curve mostly similar to that of the individual SC film; the very low slope indicative of a low viscoelastic modulus as a result of SC flexibility and ability to structurally re-adjust during the compression cycle. In spite of the similarity of the curves, the loss tangent suggests that both proteins are present in the interface (Fig. 8C). Previous work suggests that in β-casein-β-lactoglobulin blends, a primary casein layer forms at the interface while β-lactoglobulin weakly interacts as a sort of loose secondary layer (Dickinson, Rolfe, & Dalgleish, 1990), which could also be an explanation for the behavior of our 1:1 SPI-SC blend. The film made with 1:1 PPI-SC showed notably similar behavior to that of the PPI film while the 1:1 SPI-SC film appeared to be similar to the SC film (Fig. 9). PPI has a faster rate of adsorption than SPI but is slower than SC (Fig. 7D), and since displacement of SC is not likely, PPI could be filling vacant patches at the oil-water interface that were not readily occupied by SC, to form a loose outer layer.

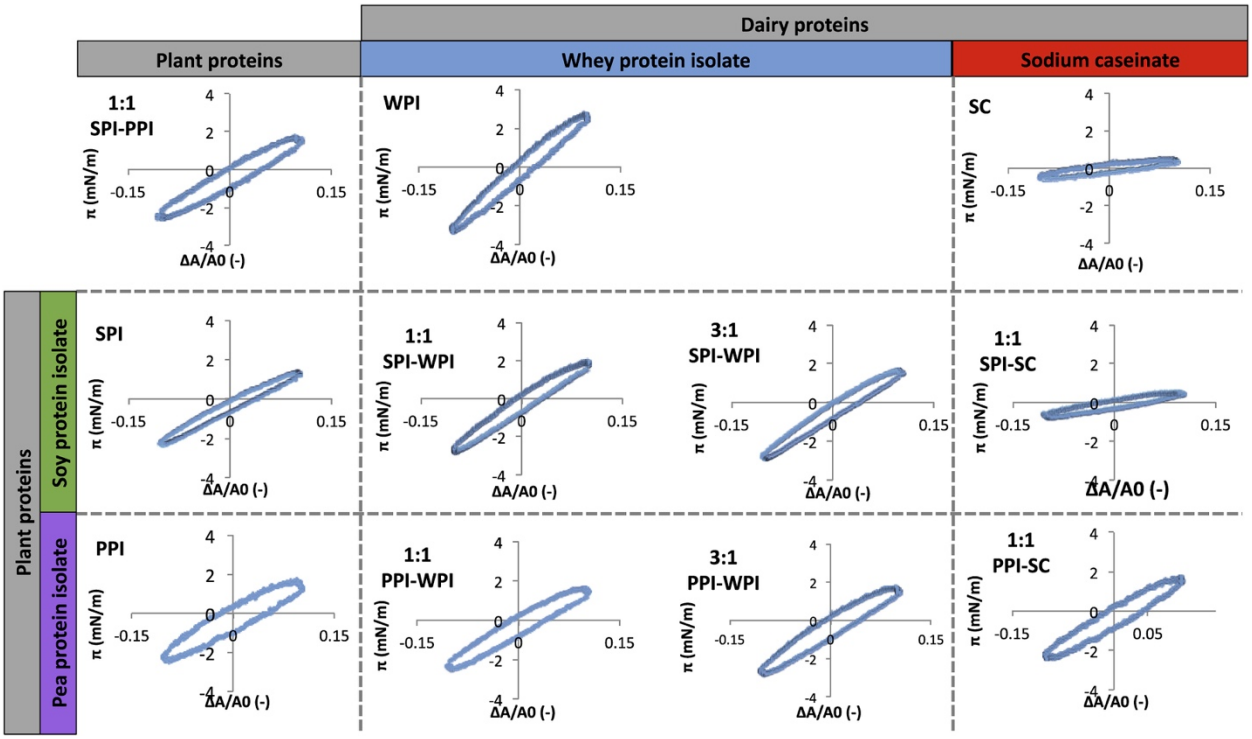

Fig. 9. Lissajous-Bowditch plots of interfacial films prepared with proteins and protein blends at the oil-water interface (amplitude = 0.1). Surface pressure (π) is plotted against the applied deformation.

Fig. 10 shows interfacial behaviour when the droplet was subjected up to 35% compression and expansion of its area, most samples showed some asymmetries: in extension (i.e., top part of the curve, from left to right), the slope of the curve is decreasing with increasing amplitude; whereas in compression (i.e., bottom part of the curve, from right to left) it is increasing with increasing amplitude. Such a shape points at strain softening and strain hardening (Berton-Carabin, Schröder, Rovalino-Cordova, Schroën, & Sagis, 2016; Van Kempen et al., 2013; Wan, Yang, & Sagis, 2016), which was most observable for the 1:1 PPI-SC blend (Fig. 10). This nonlinear interfacial behavior may indicate that distinct domains were present (Rühs, Scheuble, Windhab, & Fischer, 2013), which disconnected in extension, and jammed during compression. For SPI-WPI blends, the ratio influenced the profile of the resulting Lissajous-Bowditch plots: at 1:1 ratio (Fig. 10), the shape of the plot resembled that of WPI only whereas at 3:1 ratio, it looked more like that of SPI only, which points out that the initial amounts are of importance for the interfacial film formation and properties.

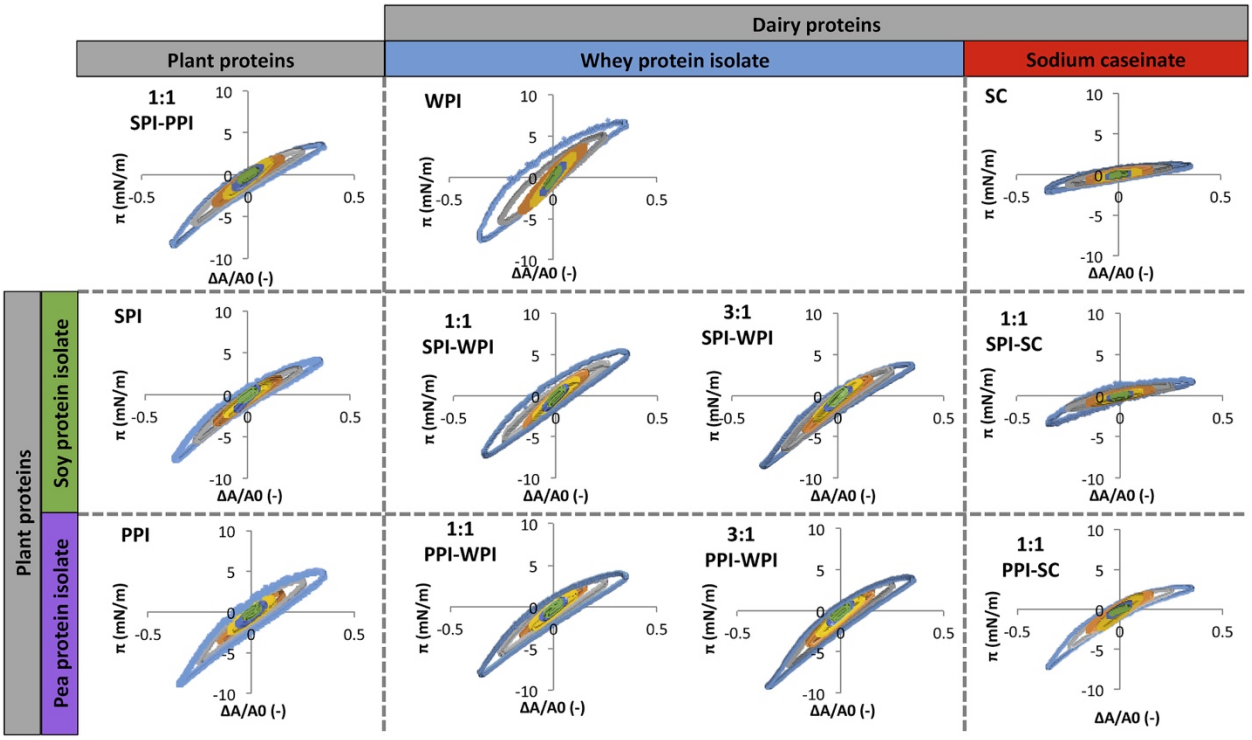

Fig. 10. Lissajous-Bowditch plots of interfacial films prepared with proteins and protein blends at the oil-water interface, at deformations (ΔA/A0 (−)) 0.03 (green), 0.05 (blue), 0.1 (yellow), 0.15 (orange), 0.25 (gray), 0.3 (light blue). Surface pressure (π) is plotted against the applied deformation.

3.3.3. Comparison of protein-protein blends and potential droplet stabilization mechanisms

Based on our findings, we have hypothesized potential organization schemas to explain the stability or instability of protein blend-stabilized emulsions (Fig. 11). All emulsions had (initially) relatively similar $d_{3,2}$ values. Emulsions stabilized with WPI-based blends exhibited enhanced physical (narrower droplet distributions) and chemical stability compared to emulsions stabilized with individual WPI or plant protein. We hypothesize that the proteins in the WPI-blends co-adsorbed and formed protein-protein interactions with adjacent proteins, to form a thick, viscoelastic protein layer. From an oxidation perspective, this co-adsorbed film could provide a protective effect through, e.g., enhanced metal or radical scavenging amino acids (Fig. 11). Alternatively, excess protein in the aqueous phase could contribute similarly as e.g. WPI has metal chelating and free radicals scavenging properties (Tong, Sasaki, Mcclements, & Decker, 2000).

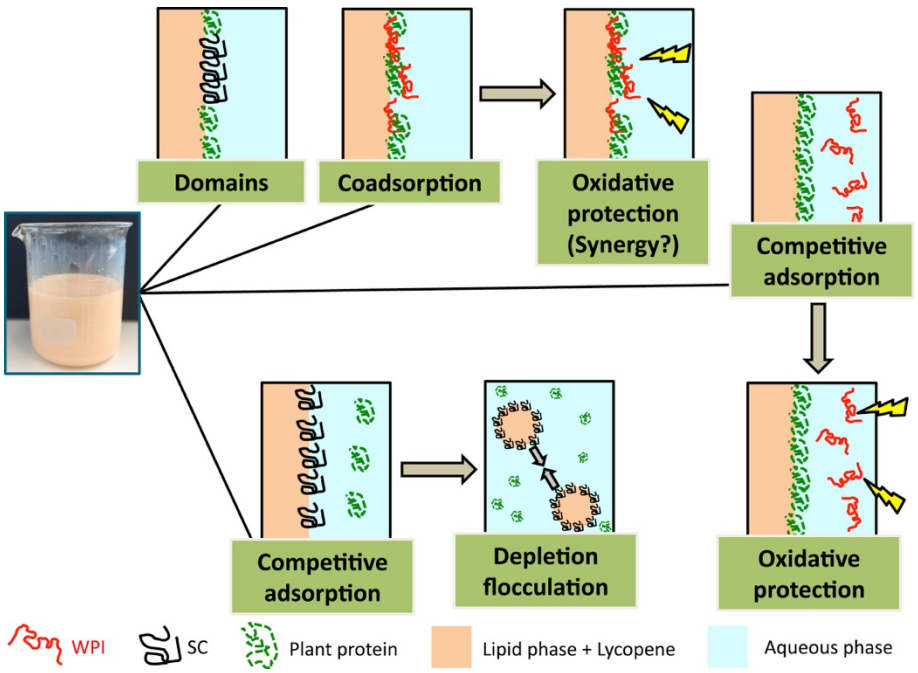

Fig. 11. Schematic of possible scenarios for protein blend behavior at the oil-water interface.

The most remarkable feature of the emulsions stabilized with SC-based blends was their rapid physical destabilization (>7 days), which is in stark contrast to the good physical stability of emulsions stabilized with SC only. Competitive adsorption may partially explain this as SC is expected to reach the interface first (Fig. 7D), unfold, and possibly exclude other proteins from the interface. As a result of this, the presence of non-adsorbed WPI, SPI, or PPI, which adsorbs at substantially slower rates (Fig. 7), could cause depletion flocculation. Aside from the differences in protein adsorption kinetics, incompatibility between proteins could explain why SC-blends exhibited depletion flocculation while others did not. Although flocculation is considered a reversible process, in unstable systems it can lead to coalescence (film rupture) and oiling off (Dickinson, 2001), which we observed as visible phase separation in the emulsions stabilized with SC-blends (Fig. 2). In this study, the emulsion protein concentration was selected to limit non-adsorbed protein (post homogenization), which makes it questionable whether sufficient protein was present in the continuous phase to induce depletion flocculation. Alternatively, it is presumable that SC rapidly formed an interfacial film around droplets during homogenization, which could have prevented further adsorption of other proteins, as previously observed by others (Aoki et al., 1984). Yet, the SC amount available for interface stabilization was 2-fold lower than in the emulsions stabilized by SC only; this could imply that in the SC-based blends, the SC amount was insufficient for long-term droplet stabilization and was not compensated by the plant protein. A third possible explanation relates to the asymmetry in the Lissajous plots observed for the 1:1 PPI-SC blend (Fig. 10), suggesting that distinct SC protein domains may exist at the interface, which could compromise film integrity and mechanical strength, due to weak packing ability and poor affinity for neighbouring proteins (Dickinson, 2001). In essence, the plot asymmetry may suggest incompatibility between SC and PPI, e.g., the formation of lateral segregated domains. Previous work on β-lactoglobulin (Lefèvre & Subirade,

2003) and globular lysozyme (Adams, Higgins, & Jones, 2002) at the oil-water interface show that globular proteins can potentially form intermolecular bonds with adjacent globular proteins at the interface. In contrast, β-casein undergoes extensive structural rearrangement (i.e., decrease in disordered structure and an increase α-helix content) upon adsorption to stabilize oil-water interfaces (Wong et al., 2012) but may be incompatible with soluble plant proteins in the mixed systems used in this study. Further work should be done to confirm interfacial protein-protein interactions for plant protein blends and incompatibility with SC when non-adsorbed protein is limited.

4. Conclusions

Our findings indicate that plant-dairy protein blends have effects that exceed those of individual proteins. Despite SC providing the best physicochemical stability of lycopene-loaded emulsions amongst individual proteins, emulsions stabilized with blends of SC and SPI or PPI were physically unstable, revealing an antagonistic effect on physical stability. Emulsions stabilized with WPI-based blends exhibited better lycopene retention compared to emulsions stabilized with individual plant proteins or WPI and lycopene retention at day 14 was the highest amongst protein blend-based emulsions.

For each blend, both proteins were introduced simultaneously to the interface, which consequently affected the ultimate rheological behavior. Proteins with the fastest adsorption rate were expected to dominate the interface, as proteins can only be displaced in very specific cases (e.g., flexible for globular proteins). Overall, blending plant proteins seemed to produce synergistic (with WPI) or antagonistic (with SC) effects on the physicochemical stability of lycopene-loaded emulsions. Although the underlying mechanisms could not be identified completely, it is clear that plant-derived emulsifiers, which are currently underutilized compared to animal-based proteins, are genuine alternatives to replace or partially replace dairy proteins for stabilization of lycopene and other lipophilic bioactives in colloidal systems.

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Appendix A. Supplementary data

Table A.1. Particle size, $d_{3,2}$ and span of lycopene-loaded emulsions over time.

Sample	$d_{3,2}$ (μm)			
	Day 0	Day 3	Day 7	Day 14
1:1 SPI-WPI	0.21 ± 0.01**a**	0.21 ± 0.01**ab**	0.22 ± 0.01**ab**	0.25 ± 0.01**a**
1:1 PPI-WPI	0.20 ± 0.00**ab**	0.20 ± 0.00**ab**	0.20 ± 0.00**bc**	0.21 ± 0.01**a**
1:1 SPI-SC	0.19 ± 0.01**bc**	0.21 ± 0.02**ab**	0.21 ± 0.02**abc**	0.29 ± 0.05**a**
1:1 PPI-SC	0.18 ± 0.00**c**	0.19 ± 0.01**b**	0.19 ± 0.02**abc**	1.17 ± 0.97**b**
3:1 SPI-WPI	0.21 ± 0.00**a**	0.23 ± 0.01**a**	0.24 ± 0.01**a**	0.23 ± 0.01**a**
3:1 PPI-WPI	0.18 ± 0.00**c**	0.20 ± 0.02**b**	0.19 ± 0.01**c**	0.20 ± 0.01**a**
1:1 SPI-PPI	0.20 ± 0.01**ab**	0.20 ± 0.01**b**	0.21 ± 0.01**bc**	0.21 ± 0.00**a**

Sample	Span			
	Day 0	Day 3	Day 7	Day 14
1:1 SPI-WPI	2.94 ± 0.21**b**	2.71 ± 0.27**cd**	2.44 ± 0.21**bcd**	2.50 ± 0.22**a**
1:1 PPI-WPI	2.21 ± 0.00**cd**	2.25 ± 0.03**de**	2.26 ± 0.02**cd**	2.17 ± 0.08**b**
1:1 SPI-SC	3.16 ± 0.14**ab**	3.00 ± 0.18**bc**	3.42 ± 0.11**ab**	11.56 ± 5.78**b**
1:1 PPI-SC	2.45 ± 0.08**c**	2.43 ± 0.02**de**	3.12 ± 1.03**abc**	3.37 ± 0.77**b**
3:1 SPI-WPI	3.08 ± 0.16**ab**	3.34 ± 0.07**ab**	3.40 ± 0.35**ab**	2.30 ± 0.31**b**
3:1 PPI-WPI	2.22 ± 0.09**cd**	2.10 ± 0.12**e**	2.17 ± 0.04**cd**	2.12 ± 0.04**b**
1:1 SPI-PPI	2.20 ± 0.04**cd**	2.16 ± 0.07**de**	2.14 ± 1.51**d**	2.16 ± 1.66**b**

Table A.2. Encapsulation efficiency (%) of lycopene-loaded emulsions fabricated with protein and protein blends.

Sample	Encapsulation efficiency (%)
WPI	58.28 ± 0.02 **abc**
SC	60.29 ± 0.01 **ab**
SPI	54.71 ± 0.04 **abcd**
PPI	63.73 ± 0.02 **a**
1:1 SPI-WPI	61.23 ± 0.09 **ab**
1:1 PPI-WPI	58.24 ± 0.03 **abc**
3:1 SPI-WPI	53.03 ± 0.03 **d**
3:1 PPI-WPI	54.75 ± 0.03 **abcd**
1:1 SPI-SC	53.03 ± 0.01 **bcd**
1:1 PPI-SC	57.05 ± 0.01**bcd**
1:1 SPI-PPI	47.91 ± 0.01 **cd**

Table A.3. Lycopene retention in emulsions stabilized with SC-blends

Sample	Lycopene Retention (%)				
	Day 3			Day 7	Day 14
1:1 SPI-SC	82.46	±	16.55	*Not measurable*	*Not measurable*
1:1 PPI-SC	73.80	±	2.92	*Not measurable*	*Not measurable*

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